

# Loss of T cell receptor-induced Bmi-1 in the KLRG1<sup>+</sup> senescent CD8<sup>+</sup> T lymphocyte

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Clones of CD8<sup>+</sup> T cells specific for viral antigens must avoid replicative senescence to maintain continuous production of new effector cells during chronic viral infections. In the present study, we have determined whether this capability may be related to Bmi-1, a transcriptional repressor that is required for the maintenance of hematopoietic stem cells and certain neural stem cells and that mediates its antisenesence function by inhibiting transcription of the *Ink4a/Arf* tumor suppressor locus. Ligand of the T cell receptor increased the levels of Bmi-1 mRNA and protein in primary CD8<sup>+</sup> T cells. The increased expression was reversible upon removal of antigen but could be maintained by using stimulation with the IL-2 receptor. Specific suppression of Bmi-1 by using a lentivirally encoded short hairpin RNA inhibited the proliferation of IL-2-stimulated CTLL-2 cytotoxic T cells and primary CD8<sup>+</sup> T cells. Ectopically expressed Bmi-1 enhanced the expansion of primary CD8<sup>+</sup> T cells stimulated by IL-2 and IL-7 *in vitro* and by homeostatic signals *in vivo*. Taken together, these findings indicate that Bmi-1 is required for CD8<sup>+</sup> T cell clonal expansion and is positively regulated by receptors that mediate this response. Therefore, the observation that the ability of the T cell receptor to induce Bmi-1 is maintained in the subset of replication-competent, antigen-experienced CD8<sup>+</sup> T cells that do not express the killer cell lectin-like receptor G1 (KLRG1) but is developmentally switched off in the senescent, KLRG1<sup>+</sup> subset suggests that Bmi-1 is a molecular determinant of the capacity of a CD8<sup>+</sup> T cell clone to persist during chronic viral infections.

Ink4a | senescence | IL-2

In the field of CD8<sup>+</sup> T cell biology, there is a need to understand how new effector CD8<sup>+</sup> T cells are generated during intermittent or persistent microbial infections because two sets of observations suggest that replicative senescence occurs in the terminally differentiated CD8<sup>+</sup> T cell. First, certain populations of antigen-experienced CD8<sup>+</sup> T cells, such as effector and effector memory cells that are CD62L<sup>low</sup> or CD127<sup>low</sup>, proliferate poorly after adoptive transfer and microbial challenge (1–7). However, this finding may be explained as the result of impaired homing of the transferred cells to appropriate sites in secondary lymphoid organs, so a second set of observations relating to human and murine CD8<sup>+</sup> T cells that express killer cell lectin-like receptor G1 (KLRG1) is especially important (8). This marker identifies effector CD8 T cells that have developed during intermittent or persistent viral infections and that proliferate poorly *in vivo* upon antigenic challenge and *in vitro* after T cell receptor (TCR) ligation (9–11). The proliferative defect was observed even in the presence of exogenous IL-2 and despite TCR-mediated IFN- $\gamma$  synthesis, indicating that the senescent phenotype is not caused by an inability to produce IL-2 or by a general defect in TCR signaling, distinguishing the KLRG1<sup>+</sup> CD8<sup>+</sup> T cell from the “exhausted” CD8<sup>+</sup> T cell in which TCR signaling is inhibited by PD-1 (12).

Although senescence in the CD8<sup>+</sup> T cell has been ascribed to telomere erosion, T cell activation can induce telomerase activity (13, 14), and replicative senescence is not prevented by expression of ectopic telomerase in human T cells (15). Another cause

of senescence may involve the stem cell-associated transcriptional repressor, Bmi-1, and the p16<sup>Ink4a</sup>/p19<sup>Arf</sup> tumor suppressor proteins. These two proteins are encoded by the *Ink4a/Arf* locus and activate the retinoblastoma protein- and p53-dependent pathways of cell cycle arrest, senescence, and apoptosis (16). Transcription of *Ink4a/Arf* is suppressed by Bmi-1 (17), a member of the Polycomb repressive complex 1 that was discovered as a cooperating oncogene in E $\mu$ -myc transgenic mice (18, 19). Mice deficient in Bmi-1 have impaired self-renewal of hematopoietic stem cells and neural stem cells (20–22), diminished T cell development in the thymus, and decreased numbers of peripheral T and B lymphocytes (23). The few mature lymphocytes that are present in *Bmi-1*<sup>−/−</sup> mice have diminished *in vitro* proliferative responses to mitogenic stimulation, reflecting either an effect of abnormal thymic development or a role for Bmi-1 in the replication of mature T cells. The abnormal lymphocyte phenotype of *Bmi-1*<sup>−/−</sup> mice is partially rescued by interruption of the *Ink4a/Arf* genes (17), with p19<sup>Arf</sup> appearing to have a more important growth inhibitory role (24). Within the hematopoietic system, Bmi-1 is most highly expressed in hematopoietic stem cells and is down-regulated upon commitment to differentiation to the Gr-1<sup>+</sup> granulocytic and Mac-1<sup>+</sup> monocytic/macrophage lineages but is maintained in mature splenic B and T lymphocytes (25). The possibility that Bmi-1 may have a role in the clonal expansion of lymphocytes is supported by the finding that ligating the B cell antigen receptor increases the expression of Bmi-1 (26).

These findings suggest that Bmi-1 may be the determinant of the replicative competence of the CD8<sup>+</sup> T cell. In the present study, we support this possibility by demonstrating that Bmi-1 is required for optimal proliferation of the CD8<sup>+</sup> T cell and that ligation of the TCR causes its expression in naïve and KLRG1<sup>−</sup> memory cells but not in senescent, KLRG1<sup>+</sup> memory cells.

## Results

**Regulation of Bmi-1 Expression in Naïve CD8<sup>+</sup> T Cells by Using Stimuli That Induce Clonal Expansion.** Bmi-1 suppresses the senescence of replicating cells (17), and it was of interest to determine whether receptors that mediate clonal expansion of the CD8<sup>+</sup> T cell induce Bmi-1 expression. We cultured purified TCR transgenic OT-I cells (27), which are specific for the ovalbumin-derived peptide OVA<sub>257–264</sub> (SIINFEKL) complexed to H-2K<sup>b</sup>, with incremental concentrations of OVA peptide for 24 h, and then we stained permeabilized cells with antibody specific for Bmi-1. The intracellular level of Bmi-1, as detected by flow cytometric analysis, increased in a dose-dependent manner, with maximal expression occurring with 2.5 nM peptide (Fig. 1*a*). The increase

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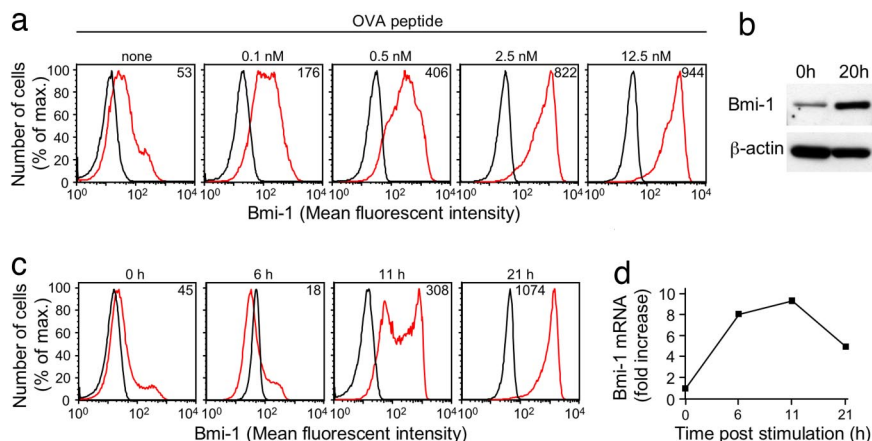
The authors declare no conflict of interest.

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Abbreviations:  $\gamma$ MHV-68, mouse  $\gamma$ -herpesvirus strain 68; KLRG1, killer cell lectin-like receptor G1; TCR, T cell receptor; shRNA, short hairpin RNA.

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**Fig. 1.** TCR signaling and induction of Bmi-1 expression in CD8<sup>+</sup> T cells. (a) Naïve OT-I T cells were cultured for 24 h in medium alone or in medium containing incremental concentrations of SIINFEKL peptide and assessed by flow cytometry for Bmi-1 expression after staining with isotype control (black line) or anti-Bmi-1 antibody (red line). (b) Whole-cell lysates of naïve OT-I cells were generated 0 and 20 h after culturing cells in medium containing 2.5 nM SIINFEKL peptide and were assessed by immunoblot for Bmi-1 and  $\beta$ -actin. (c and d) Naïve OT-I cells were cultured in the presence of 2.5 nM SIINFEKL peptide for various lengths of time and then assessed for Bmi-1 expression by flow cytometry after staining with isotype control (black line) or anti-Bmi-1 antibody (red line) (c) and assessed for Bmi-1 mRNA by quantitative RT-PCR (d). The mean fluorescent intensity for specific Bmi-1 staining is represented by the number in each histogram. These experiments were performed multiple times with comparable results.

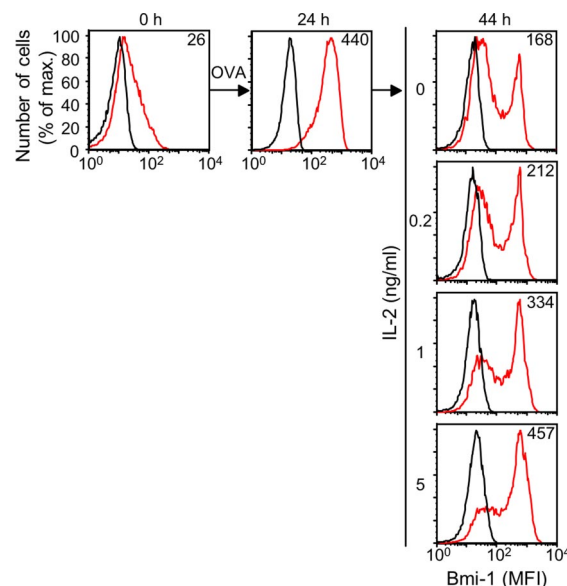
in Bmi-1 induced by 2.5 nM peptide also was demonstrated by using Western blot analysis (Fig. 1b). Addition of blocking antibodies to IL-2 and CD25 did not diminish the induction of Bmi-1 (data not shown). A kinetic analysis of TCR-induced Bmi-1 protein (Fig. 1c) and mRNA (Fig. 1d) revealed that maximal protein expression occurred by 21 h of stimulation, whereas the increase in mRNA peaked between 6 and 11 h.

Stimulation of OT-I cells with IL-2 alone did not increase expression of Bmi-1 (data not shown), as would be anticipated because resting CD8<sup>+</sup> T cells do not respond to this cytokine. However, addition of incremental concentrations of IL-2 to OT-I cells that had been cultured with 2.5 nM OVA peptide for 24 h maintained Bmi-1 expression during subsequent culture for 20 h in medium lacking antigen, which otherwise decreased in a subpopulation of cells (Fig. 2). Therefore, TCR induces expression of Bmi-1 in the naïve CD8<sup>+</sup> T cell, and this increase is maintained by using ligation of the IL-2 receptor, which is consistent with its proliferative effect not requiring repetitive TCR stimulation (28–30).

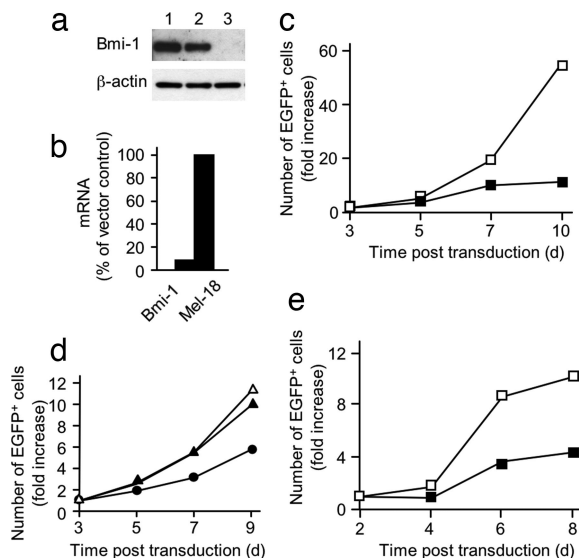
**Bmi-1 and the Proliferative Capacity of the CD8<sup>+</sup> T Cell.** T cells from Bmi-1<sup>-/-</sup> mice proliferate poorly *in vitro*, but this defect may be secondary to Bmi-1 deficiency during thymic development (23). To evaluate the effect of depleting Bmi-1 from a Bmi-1-sufficient CD8<sup>+</sup> T cell, we first validated the specificity of a lentiviral vector expressing short hairpin RNA (shRNA) by using the murine cytotoxic T cell line CTLL-2. During their IL-2-mediated proliferation, these cells were infected with lentiviral vectors expressing EGFP alone (pll3.7) or together with an shRNA that targets Bmi-1 (pll3.7-shBmi-1). The pll3.7-shBmi-1 vector suppressed both Bmi-1 protein (Fig. 3a) and mRNA (Fig. 3b), but not Mel-18 mRNA (Fig. 3b), and inhibited proliferation of the CTLL-2 cells (Fig. 3c). To exclude off-target effects of this shRNA on proliferation, we first infected replicate samples of CTLL-2 cells with a retroviral vector expressing human CD2 alone or together with a variant Bmi-1 having silent mutations in the shRNA target sequence. The CTLL-2 cells were then transduced with each of the two lentiviral vectors. In contrast to its inhibitory effect on CTLL-2 cells that had been infected with the control retrovirus, the pll3.7-shBmi-1 did not affect the proliferation of the cells expressing the mutant Bmi-1 (Fig. 3d). The rescue of the proliferation of CTLL-2 cells with the mutant

Bmi-1 excludes off-target effects of pll3.7-shBmi-1 and validates its use for analysis of primary CD8<sup>+</sup> T cells. Therefore, the inhibition of IL-2- and IL-7-mediated expansion of OT-I cells by transduction with pll3.7-shBmi-1 indicates that Bmi-1 expression is required for CD8<sup>+</sup> T cell proliferation (Fig. 3e).

The magnitude of Bmi-1 expression maintained by IL-2 receptor signaling may not be optimal for CD8<sup>+</sup> T cell proliferation, as suggested by the occurrence of a subpopulation of OT-I cells with diminished Bmi-1 in the presence of IL-2 (Fig. 2). To examine this possibility, we infected OT-I cells responding to IL-2 and IL-7 with retroviral vectors expressing rat Thy1 alone



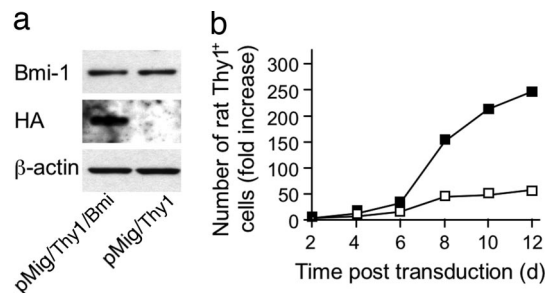
**Fig. 2.** Maintenance of Bmi-1 expression by IL-2. Naïve OT-I cells were cultured for 24 h in medium with 2.5 nM SIINFEKL peptide. The cells were then transferred to fresh medium alone or medium containing incremental concentrations of IL-2 and cultured for an additional 20 h, after which Bmi-1 expression was assessed by staining with isotype control (black line) or anti-Bmi-1 antibody (red line). The mean fluorescent intensity for specific Bmi-1 staining is represented by the number in each histogram. The experiments shown were performed multiple times with comparable results.



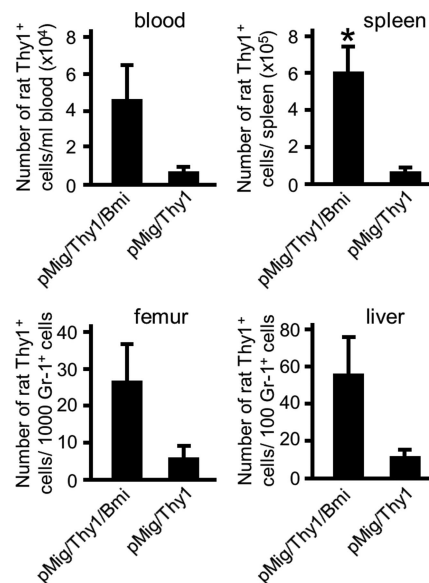
(pMig/Thy1) or with HA-tagged Bmi-1 (pMig/Thy1/Bmi-1) and measured their expansion during culture with IL-2 and IL-7 over the ensuing 12 d. Although the ectopic Bmi-1 was not overexpressed when cells were assessed on day 4 (Fig. 4a), these transduced OT-I cells expanded more effectively than did the vector control cells (Fig. 4b). We examined the capacity of ectopically expressed Bmi-1 to enhance *in vivo* expansion of the CD8<sup>+</sup> T cell by adoptively transferring OT-I cells that had been transduced with pMig/Thy1 or pMig/Thy1/Bmi-1 into Rag2<sup>-/-</sup> recipient mice and counting the number of CD8<sup>+</sup> T cells from various tissues 215 d later. There was greater expansion of the OT-I cells expressing ectopic Bmi-1 in the blood, spleen, femur, and liver (Fig. 5), indicating that augmented Bmi-1 expression enhances the response of cells to homeostatic stimuli.

### Regulation of Bmi-1 Expression in Antigen-Experienced CD8<sup>+</sup> T Cells.

The expression of KLRG1 distinguishes antigen-experienced CD8<sup>+</sup> T cells that have lost the potential for clonal expansion from cells that have retained this capability (9, 10). Because Bmi-1 regulates the proliferation of the CD8<sup>+</sup> T cell, we evaluated the possibility that loss of its TCR-induced expression may account for the replicative defect of the KLRG1<sup>+</sup> subset. We compared the expression of Bmi-1 before and 24 h after TCR ligation in naïve CD44<sup>low</sup> CD8<sup>+</sup> T cells and in the two populations of antigen-experienced CD44<sup>high</sup> cells that are distinguished by the presence or absence of KLRG1. All cells were



**Fig. 4.** Enhanced proliferation *in vitro* of CD8<sup>+</sup> T cells expressing ectopic Bmi-1. (a) OT-I cells that had been activated with anti-CD3 $\epsilon$  in the presence of IL-2 and IL-7 were transduced with pMig/Thy1 or with pMig/Thy1/Bmi-1 and maintained for 6 d in the presence of IL-2 and IL-7. On day 4 after transduction, cells expressing rat Thy1 were purified, and lysates were subjected to analysis by Western blot with antibodies specific for Bmi-1, HA, and  $\beta$ -actin. (b) In a replicate experiment, OT-I cells were transduced with pMig/Thy1 (open squares) or pMig/Thy1/Bmi-1 (filled squares) and assessed for growth in the presence of IL-2 and IL-7. The experiments shown were performed multiple times with comparable results, except for the Western blot, which was performed once.







replicative senescence of the CD8<sup>+</sup> T cell. Bmi-1 may also suppress senescence by activating telomerase, which has been reported for human mammary epithelial cells but not fibroblasts (35). If Bmi-1 does prove to be the determinant of the replicative competence of the antigen-experienced CD8<sup>+</sup> T cell, the original proposal of memory T cells needing to share certain characteristics with stem cells (36) will receive additional experimental support, as it has with the recent proposal of asymmetrical division of the antigen-stimulated CD8<sup>+</sup> T cell (37).

We do not know the signals that lead to the senescent stage of CD8<sup>+</sup> T cell development. However, there must be a subset of antigen-experienced cells that escapes these signals and maintains TCR-induced Bmi-1 expression. Because the senescent KLRG1<sup>+</sup> CD8<sup>+</sup> T cell is highly differentiated and effector differentiation is IL-2-dependent, it may be that the self-renewing subset of cells avoids replicative senescence by proliferating via an IL-2-independent pathway, such as that mediated by ligation of the TCR and CD27 (34).

## Materials and Methods

**Mice.** C57BL/6 mice were purchased from Charles River U.K. Ltd. (Margate, U.K.). OT-I RAG<sup>-/-</sup> mice and RAG2<sup>-/-</sup> mice were generous gifts from C. Reis e Sousa (Cancer Research UK London Research Institute, London, U.K.) and A. Betz (Laboratory of Molecular Biology, Cambridge, U.K.), respectively. Mice were housed under specific pathogen-free conditions at the University of Cambridge. For all animals, housing and procedures were carried out in accordance to the U.K. Home Office guidelines.

**Cell Lines and Culture Conditions.** Culture conditions and treatment of the murine B cell lymphoma line, A20 (BALB/cAnN, H-2<sup>d</sup>), and the human embryonic kidney cell line, HEK293, have been described (34). The murine cytotoxic T cell line CTLL-2 was a generous gift from J. S. Hill Gaston (Department of Medicine Addenbrooke's Hospital, University of Cambridge, U.K.) and was cultured in complete RPMI medium 1640 supplemented with 125 international units IL-2. The murine fibroblast cell line NIH 3T3 was purchased from American Type Culture Collection (Manassas, VA) and cultured in complete DMEM (34).

**Purification and Stimulation of Primary CD8<sup>+</sup> T Cells.** Naïve CD8<sup>+</sup> OT-I T cells were purified as described (34) or by negative selection with anti-biotin microbeads (Miltenyi Biotec, Auburn, CA) after binding of biotinylated antibodies against murine CD11c (N418), CD11b (M1/70), Gr-1 (RB6-8C5), MHC Class II (M5/114.15.2), CD49b (Dx-5) (eBioscience, San Diego, CA), and NK1.1 (PK136) (BD Biosciences, San Jose, CA). Cells were stimulated with anti-CD3 $\epsilon$  (clone 145-2C11) (eBioscience), either plate-bound (0.5  $\mu$ g/ml), in the presence of Fc receptor-expressing A20 cells (34), in the presence of primary B cells (1  $\mu$ g/ml), or with SIINFEKL peptide (Peptides International, Louisville, KY) (34) in the presence of mouse IL-7 (2–5 ng/ml) and IL-2 (2–5 ng/ml) (R & D Systems, Minneapolis, MN). To some cultures anti-IL-2 (clone JS6–1A12), anti-CD25 (clone PC61), and anti-CD70 (clone FR70) (eBioscience) were added.

**Viruses.** The retroviral vector pMig and the pCL-ECO packaging construct have been described previously (38). Full-length Bmi-1 cDNA was fused with a C-terminal HA-tag and inserted into the

pMig retroviral vector. For shRNA rescue experiments, Bmi-1 coding sequence was mutated at positions 458, 461, 464, 467, 470, 473, and 476 using the Gene Tailor site-directed mutagenesis system (Invitrogen, Carlsbad, CA). In the original pMig construct, EGFP served as a reporter gene. In other cases, cDNA of rat Thy1 or a truncated human CD2 (39) was inserted into pMig as alternative reporter genes. Retroviruses were produced in HEK293 cells as previously described (38). The lentiviral plasmid pLL3.7 (40) was a generous gift from Christopher P. Dillon (Massachusetts Institute of Technology, Cambridge, MA). The sequences for siRNA against Bmi-1 were obtained from Ambion (Foster City, CA) and cloned as shRNA with a loop sequence from Brummelkamp *et al.* (41) into pLL3.7. Lentiviral production and titration were performed as described (40). Primary CD8<sup>+</sup> T cells were transduced 24 h after activation in the presence of IL-2, IL-7, and 6  $\mu$ g/ml polybrene (hexadimethrine bromide; Sigma, St. Louis, MO), and IL-7 remained absent from the culture medium when CTLL-2 cells were transduced at any time.

**$\gamma$ MHV Infection.** The  $\gamma$ MHV-M3-OVA virus was a generous gift from Philip Stevenson (University of Cambridge). C57BL/6 mice were infected intranasally with  $2 \times 10^4$  pfu of  $\gamma$ MHV-M3-OVA. Splenocytes were depleted of B cells (Miltenyi Biotec), and CD8<sup>+</sup> CD44<sup>low</sup>, CD44<sup>high</sup> KLRG1<sup>-</sup>, and CD44<sup>high</sup> KLRG1<sup>+</sup> were sorted (MoFlo; Dako, Glostrup, Denmark) after staining with appropriate monoclonal antibodies.

**Assays.** Intracellular staining of Bmi-1 was performed by using the Cytofix/Cytoperm kit (BD Biosciences) and anti-Bmi-1 monoclonal antibody (1.T.21) (Abcam, Cambridge, MA) followed by anti-mouse IgG<sub>1</sub>, (A85-1) or (X56) (BD Biosciences). Staining for intracellular IFN- $\gamma$  (34) was performed after stimulation with anti-CD3 $\epsilon$  in the presence of primary B cells for 3 h. For BrdU staining, the BrdU Flow kit (BD Biosciences) was used according to the manufacturer's instructions. The antibodies used were anti-CD8a (5H10; Caltag Laboratories, Carlsbad, CA, and 53-6.7; eBioscience), anti-CD3 $\epsilon$  (145-2C11), anti-KLRG1 (2F1), anti-CD44 (IM7), anti-Thy1.1 (HIS51; eBioscience), anti-Gr-1 (RB6-8C5), and anti-CD2 (S5.2; BD Biosciences).

For quantitative RT-PCR analysis primers and TaqMan probes for detection of murine Bmi-1, CD3 $\epsilon$ , and Mel-18 mRNA were purchased from Applied Biosystems (Foster City, CA). The sequences of the primers and probes for p16<sup>Ink4a</sup> and p19<sup>Arf</sup> used were as follows: p19<sup>Arf</sup>, 5'-gccgcaccggaatctt-3' (sense); p19<sup>Arf</sup>, 5'-ttgagcagaagagctgctacgt-3' (antisense); p19<sup>Arf</sup>, 5'-accagtgatgatgatgggcaact-3' (probe); p16<sup>Ink4a</sup>, 5'-cgtaccgccgattcagtgat-3' (sense); p16<sup>Ink4a</sup>, 5'-gggtctctcgagtcgaa-3' (antisense); and p16<sup>Ink4a</sup>, 5'-cgttcacgtacgactctcttctcaact-3' (probe).

The antibodies used for immunoblot analysis of Bmi-1 expression were mouse anti-Bmi-1 antibody [1.T.21] (Abcam), goat anti-mouse IgG<sub>1</sub>-HRP (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti- $\beta$ -actin (Abcam), goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology), and mouse anti-MonoHA 1.1 (Covance, Princeton, NJ).

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